Journal of Chromatography, 570 (1991) 166–172
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 5964

Short Communication

Indolic urinary melanogens: separation and identification by gas chromatography with selected-ion monitoring mass spectrometry of 5-hydroxy-6-methoxyindole-2-carboxylic and 5-methoxy-6-hydroxyindole-2-carboxylic acids

J. M. DUTHEL* and J. J. VALLON

Laboratory of Biochemistry, Toxicology and Trace Analysis (Professor J. J. Vallon), Edouard Herriot Hospital, Place d'Arsonval, F-69437 Lyon Cedex 03 (France)

(First received January 23rd, 1991; revised manuscript received April 30th, 1991)

ABSTRACT

Two isomeric urinary melanogens, 5-hydroxy-6-methoxyindole-2-carboxylic acid and 5-methoxy-6-hydroxyindole-2-carboxylic acid, have been separated by gas chromatography with selected-ion monitoring mass spectrometry. After chemical synthesis of one of these two isomers, 5-methoxy-6-hydroxyindole-2-carboxylic acid, and the establishment of the mass spectrum of its trimethylsilylated derivative, a 30-ml sample of a melanotic 24-h urine was adjusted to pH 1 and extracted twice with 10 ml of ethyl acetate. The extract was evaporated to dryness and the residue derivatized with methyl-8, followed by Tri-Sil/TBT. Silylated derivatives were analysed by gas chromatography with selected-ion monitoring mass spectrometry. The mass spectrum of the 5-methoxy-6-hydroxyindole-2-carboxylic acid allowed the determination of the retention times of both isomers.

INTRODUCTION

Melanin formation, and especially eumelanin biosynthesis in malignant melanoma, has been the subject of in depth studies [1–8] for *ca.* sixty years. Owing to the complexity of the matrix and the presence of numerous melanin precursors and their metabolites, some of them are difficult to separate by chromatography. The three main separation techniques currently used for indolic derivatives are thin-layer chromatography (TLC), high-performance liquid chromatography (HPLC) and gas chromatography (GC) [9]. The melanogen system is composed of specialized pigment cells, melanocytes, inside which a specific enzyme, tyrosinase, converts tyrosine into dopa and then into dopaquinone, which is cyclized and oxidized to dopachrome. Decarboxylation of dopachrome leads to 5,6-dihydroxyindole (5,6-DHI); oxidation of 5,6-dihydroxyindole gives melanin. In melanoma, 5,6-dihydroxyindole-2-carboxylic acid is derived from dopachrome and

0378-4347/91/$03.50 © 1991 Elsevier Science Publishers B.V.

gives two methylated isomers, which are eliminated in the urine: 5-hydroxy-6-methoxyindole-2-carboxylic acid (5-H-6-MI-2-C acid; so called C_{4a}, compound No. 4, a-isomer), and 5-methoxy-6-hydroxyindole-2-carboxylic acid (5-M-6-HI-2-C acid; C_{4b}, compound No. 4, b-isomer). Three other unconjugated melanogens found in urine were called [6] C_1, C_2 and C_3. This paper describes the separation, by gas chromatography (GC) with selected-ion monitoring mass spectrometry (SIM-MS), of these two urinary indolic melanogens for diagnostic purposes.

EXPERIMENTAL

Reagents

The materials and reagents necessary for melanogen extraction are described in the method of Armstrong *et al.* [10]. DL-Dopa, 6-benzyloxy-5-methoxyindole-2-carboxylic acid, 5-hydroxyindole-3-acetic acid (5-HIAA), 5-hydroxyindole-2-carboxylic acid (5-HICA), β-(4-hydroxy-3-methoxyphenyl)lactic acid (C_2), DL-*p*-hydroxyphenyllactic acid (C_3), indole-2-carboxylic acid (I-2-CA), 5-hydroxyindole (5-HI) and 5-methoxyindole (5-MI) were purchased from Sigma (St. Louis, MO, USA); methyl-8 and Tri-Sil/TBT were obtained from Pierce (Rockford, IL, USA).

Apparatus

A gas chromatograph–mass spectrometer, QP-1000 (Shimadzu, Tokyo, Japan), with an automatic sample injector (AOC-9, Shimadzu) was used. The computer for acquisition and processing of data was a V 286 (Victor Technologies, Stockholm, Sweden), connected to a printer, KX-P 1083 (Panasonic, Matsushita Electric Trading, Osaka, Japan).

Gas chromatography

The wide-bore borosilicate glass column was an SPB-1 (30 m × 0.75 mm I.D. 1.0 μm film thickness, Supelco, Bellefonte, PA, USA); carrier gas, helium N 60 (Air Liquide, Paris, France); column head pressure, 0.20 bar; splitless injector temperature, 260°C; GC oven temperature, initially 140°C for 2 min then programmed at 6°C/min to 260°C then held for 2 min; total cycle time, 31 min.

Mass spectrometry

Electron-impact (EI) ionization with 70 eV energy and an emission current of 60 μA was used; temperature of transfer line and jet separator, 280°C; ion source temperature, 250°C; make-up gas flow-rate, 20 ml/min; the data were obtained in scan mode from 49 to 750 atomic mass units at 1.4 s per cycle, or in the SIM mode; the instrument was autotuned with perfluorotributylamine (PFTBA); the electron multiplier voltage was 1400 V.

Synthesis of one of the indolic melanogens

5-M-6-HI-2-C acid was prepared following the method of Wakamatsu and Ito [11], which is a debenzylation of 6-benzyloxy-5-methoxyindole-2-carboxylic acid by catalytic hydrogenation.

Urinary extraction

The 24-h urine sample was collected with toluene, then stored and frozen at −20°C in the dark. Ethyl acetate extraction (pH 1) followed the method of Armstrong *et al.* [10].

Derivatization

The ethyl acetate extract (1 ml) was transferred to a 5-ml PTFE-lined screw-capped vial, then evaporated at room temperature under a stream of nitrogen. The residue was redissolved in 400 μl of methyl-8, vortex-mixed to aid dissolution, then sonicated and finally incubated at 60°C for 15 min in a dry block heater. After this time the content of the vial was evaporated at room temperature under a stream of nitrogen; the residue was again redissolved in 400 μl of Tri-Sil/TBT, vortex-mixed, then sonicated and finally incubated at 70°C for 30 min in a dry block heater. The vial was again evaporated at room temperature under a stream of nitrogen. The sample was finally reconstituted with 200 μl of hexane, and 1 μl was injected into the gas chromatograph–mass spectrometer. The derivatization of indolic standards, and notably of 5-M-6-HI-2-C acid, includes the same steps as for the urinary extract.

RESULTS AND DISCUSSION

Fig. 1 shows the total ion chromatogram of the mono- or bis-trimethylsilylated (TMS) derivatives of seven aromatic compounds which, in elution order, are C_2-$Si(CH_3)_3$,5-HI-$Si(CH_3)_3$,5-MI-$Si(CH_3)_3$,I-2-CA-$Si(CH_3)_3$,5-HI-$[Si(CH_3)_3]_2$, C_3-$[Si(CH_3)_3]_2$, C_2-$[Si(CH_3)_3]_2$, 5-HICA-$[Si(CH_3)_3]_2$, 5-HIAA-$[Si(CH_3)_3]_2$ and 5-HIAA-$[Si(CH_3)_3]_3$. The interpretation of the various O-TMS derivative mass spectra, and especially of 5-HICA-$[Si(CH_3)_3]_2$ (Fig. 2), whose structure is very similar to that of the O-TMS derivative of C_{4a} or C_{4b}, has enabled us to elucidate the fragmentation of C_{4b} as a double silylation with major ions *m/z* 73, 231, 321 and 351.

Synthesis of C_{4b} was achieved by debenzylation of 6-benzyloxy-5-methoxyindole-2-carboxylic acid; it was then derivatized with methyl-8 followed by Tri-Sil/TBT and chromatographed. The mass spectrum of the product appeared to show the same ions as those obtained by calculation; this mass spectrum is characteristic of the bis-silylated compound of C_{4b}. The retention time of this isomer (14.24 min) has been used for the identification of the peak in the analysis of the urine extract.

For the identification of both isomers (C_{4a} and C_{4b}), a 24-h urine sample of a

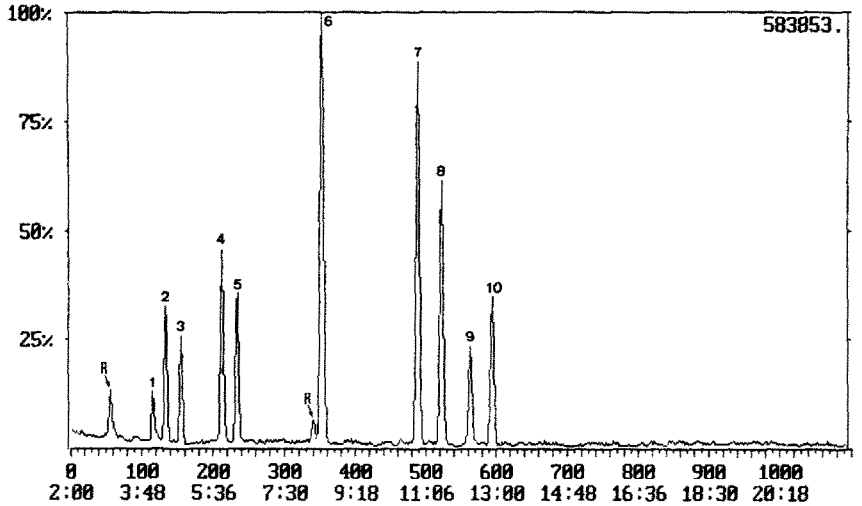

Fig. 1. Total ion chromatogram (EI 70 eV) for a mixture of O-TMS derivatives of seven aromatic compounds (50 ng of each component). Peaks: R = trimethylsilylation reagent; 1 = C_2-Si$(CH_3)_3$; 2 = 5-HI-Si$(CH_3)_3$; 3 = 5-MI-Si$(CH_3)_3$; 4 = I-2-CA-Si$(CH_3)_3$; 5 = 5-HI-[Si$(CH_3)_3]_2$; 6 = C_3-[Si$(CH_3)_3]_2$; 7 = C_2-[Si$(CH_3)_3]_2$; 8 = 5-HICA-[Si$(CH_3)_3]_2$; 9 = 5-HIAA-[Si$(CH_3)_3]_2$; 10 = 5-HIAA-[Si$(CH_3)_3]_3$.

melanoma patient with a Thormahlen-positive reaction was extracted, derivatized, then chromatographed (Fig. 3) under the same conditions as previously. The mass spectrum (Fig. 4) and the retention time (14.24 min) allowed identification of C_{4b} in urine. The closest peak (14.12 min) gives the same mass spectrum as C_{4b}, except for the differences in the minor relative abundance (Fig. 5); this is the mass spectrum of the bis-TMS derivative of the C_{4b} isomer, the C_{4a}-bis-TMS or 5-H-6-MI-2-C-[Si$(CH_3)_3]_2$. Fig. 4 and 5 illustrate the expected mass of ion fragments *m/z* 73, 231, 261 and the molecular ion *m/z* 351. The *m/z* 73 fragment is characteristic of the TMS group. The difference between fragments *m/z* 261 and 351 corresponds to the loss of $(CH_3)_3SiOH$. Fragments *m/z* 336, 321 and 306 correspond to the loss of methyl groups (*m/z* 15), and the difference (*m/z* 30)

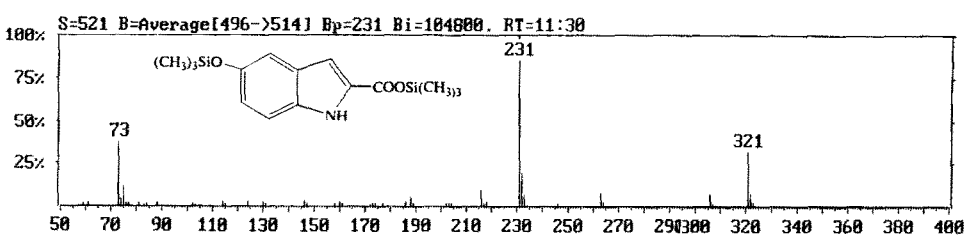

Fig. 2. Mass spectrum (EI 70 eV) and structure of the bis-TMS derivative of 5-HICA or 5-HICA-[Si$(CH_3)_3]_2$.

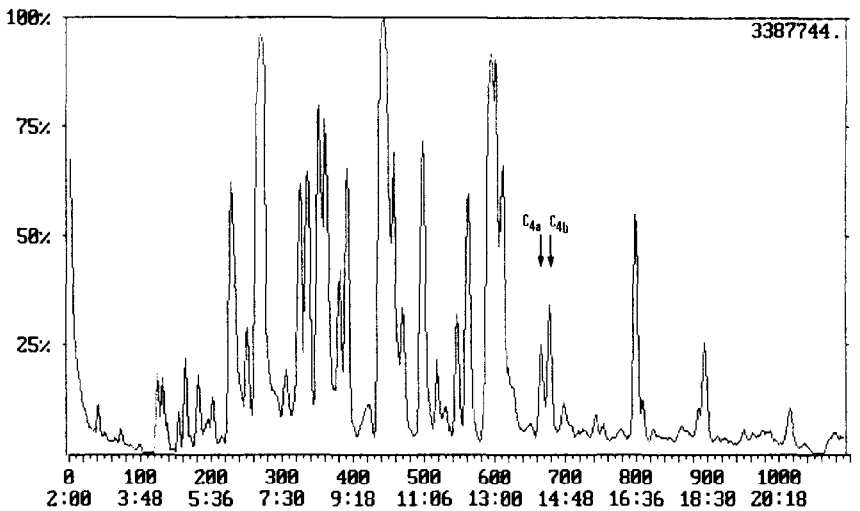

Fig. 3. Total ion chromatogram (EI 70 eV) of urinary extract from a melanoma patient showing the separation of C_{4a} and C_{4b}.

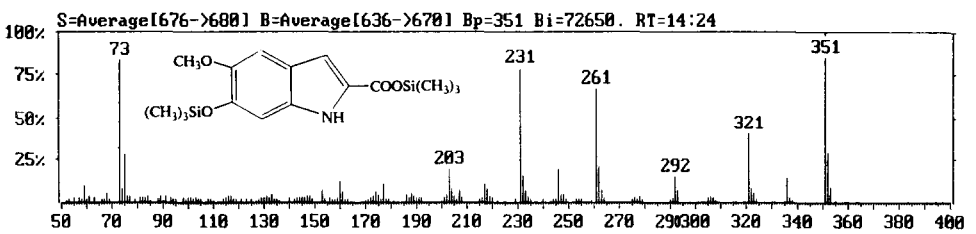

Fig. 4. Mass spectrum (EI 70 eV) and structure of the bis-TMS derivative of C_{4b} or 5-M-6-HI-2-C-$[Si(CH_3)_3]_2$.

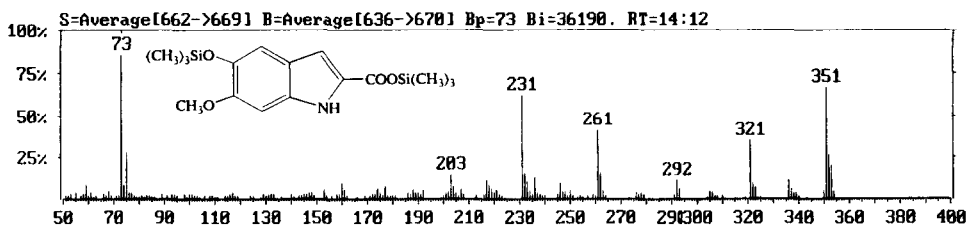

Fig. 5. Mass spectrum (EI 70 eV) and structure of the bis-TMS derivative of C_{4a} or 5-H-6-MI-2-C-$[Si(CH_3)_3]_2$.

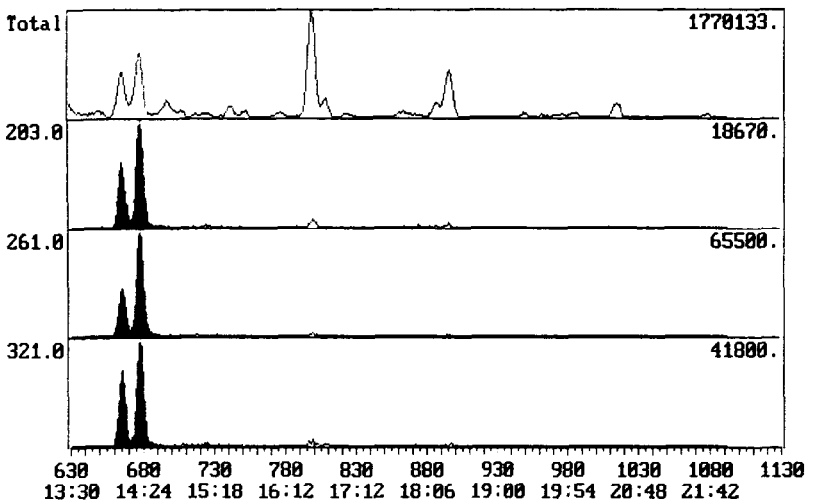

Fig.6. SIM of C_{4a} and C_{4b} with *m/z* 203, 261, 321 and partial total ion chromatogram of Fig. 3, showing the separation of the two isomers.

between fragments *m/z* 231 and 261 corresponds to the loss of CH_2O. Compared with other compounds in the chromatogram (Fig. 3) a very low urinary concentration of both isomers was found, so that the total ion chromatogram shows numerous important peaks due to the relatively important sampling (2.5 μl). Nevertheless, the SIM of ions *m/z* 203, 261 and 321 (Fig. 6) shows that the separation of the two isomers is very satisfactory. This method does not require previous HPLC purification as proposed in a previous report [7].

In conclusion, this method with optimized conditions for derivatization and chromatography allows good separation and reliable identification of the two indolic isomers of urinary melanogens, the C_{4a} and C_{4b}. A future study will examine the quantification of both isomeric melanogens and will measure the variability of their ratio in normal and melanoma patients.

ACKNOWLEDGEMENT

The authors thank the Industrial Physical and Chemical Institute of Lyon (Lyon, France) for the synthesis of the 5-methoxy-6-hydroxyindole-2-carboxylic acid.

REFERENCES

1 J. Duchon and Z. Pechan, *Ann. N.Y. Acad. Sci.*, 100 (1963) 1048.
2 J. D. Benigni and R. L. Minnis, *J. Heterocyclic Chem.*, 2 (1965) 387.
3 J. Duchon and B. Matous, *Clin. Chim. Acta*, 16 (1967) 397.
4 A. Badinand, J. Pasquier, J. J. Vallon, R. Guilluy and H. Milon, *Clin. Chim. Acta*, 25 (1969) 357.

5 J. J. Vallon, *Lyon Pharm.*, 23 (1972) 543.
6 J. J. Vallon, A. Badinand and C. Bichon. *Clin. Chim. Acta*, 36 (1972) 397.
7 C. Hansson, *Acta Derm. Venereol.*, 64 (1984) 185.
8 M. C. Ekelund, R. Carstam, C. Hansson, H. Rorsman and E. Rosengren, *Acta Derm. Venereol.*, 65 (1985) 437.
9 P. M. M. Van Haard and S. Pavel, *J. Chromatogr.*, 429 (1988) 59.
10 M. D. Armstrong, N. Kenneth, F. Shaw, M. J. Gortatowski and H. Singer, *J. Biol. Chem.*, 232 (1958) 17.
11 K. Wakamatsu and S. Ito, *Anal. Biochem.*, 170 (1988) 335.